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Inhibition of MMP-2 activation and release as a novel mechanism for HDL-induced cardioprotection

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Abstract **High density lipoproteins (HDL) protect the heart against ischemia/reperfusion (I/R) injury, and matrix metalloproteinase-2 (MMP-2) directly contributes to cardiac contractile dysfunction after I/R. To investigate the possible involvement of MMP-2 inhibition in HDL-mediated cardioprotection, isolated rat hearts underwent 20 min of low-flow ischemia and 30 min of reperfusion. Plasma-derived and synthetic HDL attenuated the I/R-induced cardiac MMP-2 activation and release in a dose-dependent way. The attenuation of I/R-induced MMP-2 activation by HDL correlated with the reduction of post-ischemic contractile dysfunction and cardiomyocyte necrosis. These results indicate prevention of MMP-2 activation as a novel mechanism for HDL-mediated cardioprotection.**

Keywords: High density lipoproteins; Gelatinase A; Matrix metalloproteinases; Myocardial ischemia; Ischemia/reperfusion injury

1. Introduction

Matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases involved in the remodeling of extracellular matrix (ECM). They are synthesized and secreted as pro-enzymes (zymogens), and then activated by proteinases or by non-proteolytic agents that can modify the "cysteine switch" [1]. MMP-2, or gelatinase A, is expressed in the myocardium, where it is synthesized by fibroblasts and cardiomyocytes [2]. Within the heart, MMP-2 degrades almost all ECM components and participates in the long-term cardiac remodeling which follows a myocardial infarction [2]. Indeed, targeted deletion or pharmacological inhibition of MMP-2 improves cardiac activity and survival rate after acute myocardial infarction [3]. Recently, a series of non-ECM substrates for MMP-2 has been described; among these, troponin I (TnI) [4], thus implying MMP-2 as a primary mediator of cardiac dysfunction following I/R [5].

We have recently demonstrated that plasma-derived or synthetic HDL cause a rapid improvement of post-ischemic cardiac function in isolated rat hearts undergoing ischemia/reperfusion (I/R) injury; this cardioprotective effect is dose-dependent and occurs at HDL concentrations that are normally found in the plasma of healthy individuals [6,7].

HDL have been shown to inhibit the expression and activation of MMP-2 and MMP-9 induced by oxidized LDL in smooth muscle cells and macrophages [8,9]. Moreover, the infusion of plasma-derived and synthetic HDL prevented MMP-9 expression in rabbit aortic lesions induced by a combination of cholesterol rich diet and balloon denudation [10]. The aim of the present study was thus to investigate whether a modulation of MMP-2 expression and/or activation could contribute to the cardioprotective effect of plasma-derived and synthetic HDL in isolated hearts undergoing I/R injury.

2. Materials and methods

2.1. Experimental protocol

I/R was carried out in isolated rat hearts as previously described [6,7]. Briefly, hearts were equilibrated with Krebs–Henseleit (K–H) buffer at a flow rate of 15 ml/min for 30 min and underwent a 20 min low-flow ischemia (1 ml/min) followed by 30 min of reperfusion at the starting flow. Human plasma HDL, low density lipoproteins (LDL) and synthetic HDL (sHDL) were prepared as previously described [6,7], and administered during the 10 min immediately before ischemia ($n = 3$ for each treatment group); a separate group of hearts was similarly treated with an equal volume of saline. Control hearts ($n = 3$) were mounted and perfused with K–H buffer at the constant flow rate of 15 ml/min for 80 min. At the end of the experiment, hearts were snap-frozen in liquid nitrogen and stored at −80 °C. Cardiac homogenates were prepared as described [6] and suspended in PBS containing AEBSF 2 mM, EDTA 1 mM, Bestatin 130 μM, E-64 14 μM, Leupeptin 1 μM, Aprotinin 0.3 μM (Sigma–Aldrich). After 1 h incubation at 4 °C, the homogenates were centrifuged for 20 min at 20 000 × *g* at 4 °C and protein content measured by the BCA method (Pierce).

2.2. Zymography and Western blotting for MMP-2

The gelatinolytic activity of cardiac homogenates (40 μg of total protein) was evaluated by zymography, as previously described [11]. Band densities were evaluated with a GS-690 Imaging Densitometer and a Multi-Analyst software (Bio-Rad Laboratories). Aliquots of coronary effluent (10 ml) collected in the first 5 min of reperfusion were

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Abbreviations: ApoA-I, apolipoprotein A-I; CK, creatine kinase; ECM, extracellular matrix; FN, fibronectin; HDL, high density lipoproteins; I/R, ischemia/reperfusion; K–H, Krebs–Henseleit; LDL, low density lipoproteins; LOOH, lipid hydroperoxide; LVDP, left ventricular developed pressure; MMP-2, matrix metalloproteinase 2; ROS, radical oxygen species; sHDL, synthetic HDL; TnI, troponin I

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lyophilized and subjected to SDS–PAGE; the separated proteins were transferred on nitrocellulose membranes. Membranes were saturated with 5% non-fat dried milk at 4 °C, and incubated with a mouse monoclonal antibody against MMP-2 (Calbiochem) and with an HRP-conjugated rabbit anti-mouse antibody (Dakocytomation). Bands were visualized by enhanced chemiluminescence (Amersham Biosciences). Each experiment was performed at least three times with different heart preparations.

2.3. Western blotting for fibronectin

Cardiac homogenates (20 μg of total protein) were subjected to SDS-PAGE (8% acrylamide) under reducing conditions and the separated proteins were transferred on nitrocellulose membranes. Membranes were saturated overnight with 5% non-fat dried milk at 4 °C, incubated with a rabbit polyclonal antibody against rat fibronectin (Sigma–Aldrich) and with an HRP-conjugated goat anti-rabbit antibody (Dakocytomation). Bands were visualized by enhanced chemiluminescence (Amersham Biosciences). Membranes were then stripped and reprobed with an antibody against α-actin (Sigma–Aldrich).

2.4. RT-PCR

Total RNA was extracted from cardiac homogenates with Trizol Reagent (Life Technologies); cDNA was prepared by reverse transcription of 1 μg total RNA using the iScript cDNA Synthesis kit (Bio-Rad Laboratories), and amplified for 30 cycles with *iTaq* DNA polymerase (Bio-Rad laboratories) in a MyCycler (Bio-Rad Laboratories). The following primers were used: MMP-2 sense 5′-CCCCTATCTACACCTACACCAAGAAC-3′, antisense 5′-CATTCCAGGAGTCTGCGATGAGC-3′, producing a 576 bp fragment; GAPDH sense 5′-ACGACCCCTTCATTGACC-3′, antisense 5′-TGCTTCACCACCTTCTTG-3′, producing a 691 bp fragment. PCR products were separated by electrophoresis on 1% agarose gel, stained with ethidium bromide, visualized by ultraviolet irradiation and photographed with Polaroid film. Band densities were evaluated with a GS-690 Imaging Densitometer and a Multi-Analyst software (Bio-Rad Laboratories). MMP-2 band intensities were normalized by their GADPH values.

2.5. Determination of cardiac lipid hydroperoxides

Aliquots of cardiac homogenates were suspended in PBS containing 5 mM BHT and centrifuged at 4 ° C for 10 min at 20000 × *g*. Protein concentration was measured by the method of Lowry [12]. Lipid hydroperoxide (LOOH) concentration was estimated by a iodometric procedure: after incubation with phosphate-buffered potassium iodide (0.12 M) in the dark for 30 min, the conversion of iodide to iodine by LOOH was measured at 365 nm. Concentrations of LOOH in cardiac homogenates were calculated by the molar absorption of I_3 at 365 nm (24,600 M^{-1} cm^{-1}) and corrected for protein concentration.

2.6. Statistical analysis

Results are reported as mean ± SEM. Differences among the groups were evaluated by 1-way ANOVA, with post hoc evaluation by the Neuman–Keuls test. Statistical significance was defined as $P < 0.05$. Simple regression analyses were performed and significance of the correlation was determined by the Pearson method.

3. Results

3.1. HDL attenuate cardiac MMP-2 activation and release

Gelatinolytic activities in homogenates of non-ischemic (Control) hearts were detected by zymography at 75, 72 and 62 kDa (Fig. 1). The 72 kDa and the 62 kDa bands were identified as proMMP-2 and MMP-2, respectively. It is important to note that in the SDS-containing gels, and differently from the in vivo condition, both latent and active MMP-2 display gelatinolytic activity. As expected, proMMP-2 represented the major gelatinolytic activity in control hearts. I/R caused an almost complete loss of both MMP-2 forms, as shown by the clear-cut reduction of gelatinolytic activity in saline-treated hearts (Fig. 1). In parallel, both pro- and active MMP-2 were

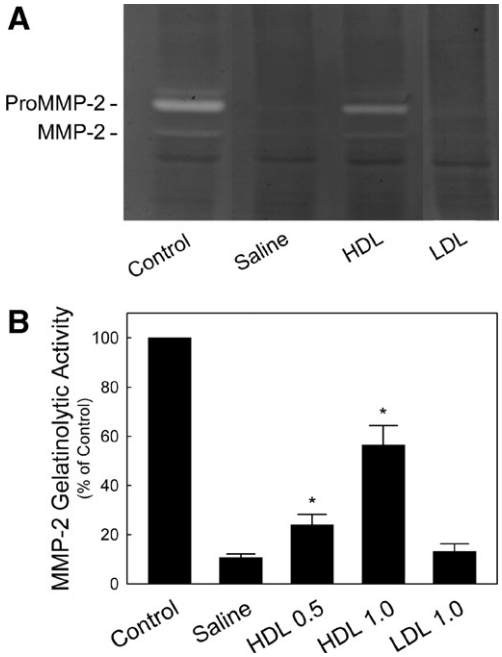

Fig. 1. Effect of HDL on cardiac MMP-2 content. Panel A: Representative zymography of homogenates from non-ischemic hearts (Control) and I/R hearts treated with saline, HDL or LDL (both at 1 mg of protein/ml) during the 10 min immediately before ischemia. Panel B: Densitometric analysis of total MMP-2 gelatinolytic activity in cardiac tissue samples. Data are expressed as mean ± SEM, $n = 3$ for each group. $^{*}P < 0.05$ versus saline.

detected in the coronary effluent collected during the first 5 min of reperfusion (Fig. 2). Active MMP-2 was the predominant form in the coronary effluent, indicating that I/R actually caused the activation and release of cardiac MMP-2. The degradation of fibronectin, one of the non-collagenous components of ECM and substrate of MMP-2 [3], confirmed that MMP-2 is activated in ischemic hearts (Fig. 3).

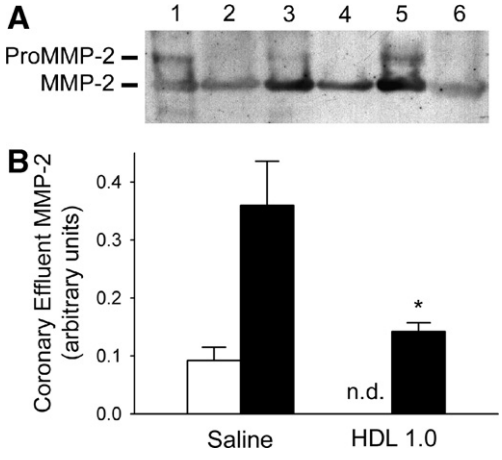

Fig. 2. MMP-2 content in the coronary effluent. Panel A: Immunoblotting analysis for MMP-2 of coronary effluents from I/R hearts treated with saline (lane 1, 3 and 5) or HDL at 1 mg/ml (lane 2, 4 and 6). Panel B: Densitometric analysis of proMMP-2 (white bars) and active MMP-2 (black bars) bands. ProMMP-2 was undetectable (n.d.) in the coronary effluent of HDL-treated hearts. Data are mean ± SEM, $n = 3$ for each group. $^{*}P < 0.05$ versus saline.

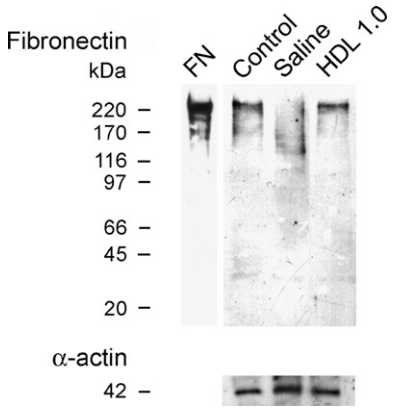

Fig. 3. Effect of HDL on fibronectin degradation. Immunoblotting of homogenates from non-ischemic hearts (Control, lane 2) and I/R hearts treated with saline (lane 3) or with plasma-derived HDL (1 mg of protein/ml, lane 4) immediately before ischemia. Purified fibronectin was also immunoblotted as control (FN, lane 1). Immunoblotting for α-actin was used to confirm the application of similar amount of proteins to each lane. Representative of three separate experiments.

HDL given immediately before ischemia attenuated in a dose-dependent way the I/R-induced loss of cardiac gelatinolytic activity, without affecting the proMMP-2/active MMP-2 ratio (Fig. 1); at the maximum HDL dose, cardiac total gelatinolytic activity was 5.3 fold higher than in saline-treated I/R hearts (Fig. 1). ProMMP-2 was undetectable in the coronary effluent of HDL-treated hearts and active MMP-2 was significantly lower than in coronary effluent of saline-treated hearts ($P = 0.048$) (Fig. 2), indicating that HDL prevented I/R-nduced MMP-2 activation and release. Indeed, fibronectin degradation was significantly attenuated in HDL-treated I/R hearts (Fig. 3). The preservation of cardiac MMP-2 content paralleled the HDL-induced prevention of postischemic cardiac dysfunction and damage [6], as indicated by the significant correlation between total cardiac MMP-2 and post-ischemic LVDP recovery ($R = 0.953$, $P < 0.001$), and CK release ($R = -0.954$, $P < 0.001$). LDL, which do not possess cardioprotective activity [6], did not preserve cardiac MMP-2 content, as demonstrated by the absence of gelatinolytic activity in LDL-treated hearts (Fig. 1). sHDL made of apolipoprotein A-I (apoA-I) and egg phosphatidylcholine also preserved cardiac MMP-2 content (Fig. 4), which again correlated with post-ischemic LVDP recovery ($R = 0.998$, $P = 0.002$) and CK release ($R = -0.985$, $P = 0.015$) [7]. Lipid-free apoA-I had a lower but significant effect on cardiac MMP-2 content (Fig. 4). MMP-2 mRNA levels did not differ among control, saline-, HDL-, and LDL-treated hearts (Fig. 5), indicating that the differences in cardiac MMP-2 content observed among the various groups of hearts were not the result of changes in MMP-2 gene expression.

3.2. HDL reduce cardiac LOOH levels

Radical oxygen species (ROS) can trigger the activation of latent MMPs by oxidative modification of the "cysteine switch" [1,13]. To evaluate whether HDL inhibition of MMP-2 activation and release was due to a reduction of ROS levels, the concentrations of LOOH, the major initial reaction products of lipid peroxidation, were measured in cardiac tissues. If compared to non-ischemic control hearts, I/R hearts treated with saline displayed a 4.3 fold increase of

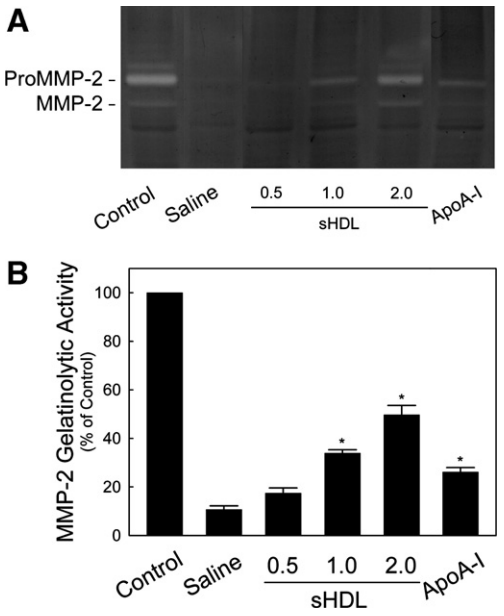

Fig. 4. Effect of sHDL on cardiac MMP-2 content. Panel A: Representative zymography of homogenates from non-ischemic hearts (Control) and I/R hearts treated with saline, sHDL (0.5–2.0 mg of protein/ml), or apoA-I (1 mg/ml) during the 10 min immediately before ischemia. Panel B: Densitometric analysis of total MMP-2 gelatinolytic activity in cardiac tissue samples. Data are expressed as mean ± SEM, $n = 3$ for each group. $^{*}P < 0.05$ versus saline.

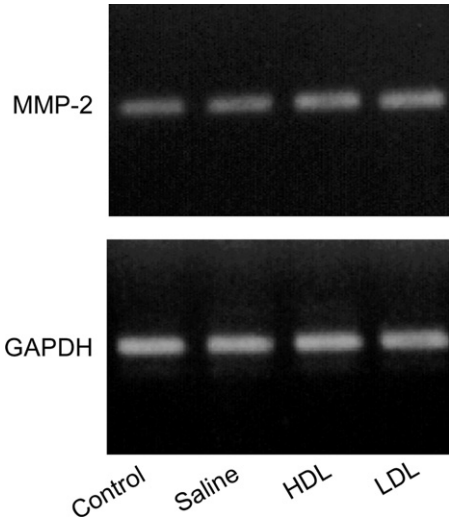

Fig. 5. Effect of HDL on cardiac MMP-2 expression. RT-PCR analysis for MMP-2 and GAPDH of non-ischemic (Control) hearts and I/R hearts treated with saline, HDL or LDL (both at 1 mg of protein/ml). Representative of three separate experiments.

LOOH levels ($P < 0.001$ vs controls) that was significantly reduced by the treatment with HDL: at the maximum dose of HDL (1 mg/ml), cardiac LOOH concentration was reduced by 58.6% ($P < 0.001$ vs saline-treated hearts) (Fig. 6). sHDL also inhibited I/R-induced LOOH formation in a dose-dependent way, with a 57.2% reduction at the highest dose (2 mg/ml) (Fig. 6). The treatment with LDL did not significantly affect I/R-induced LOOH formation, while lipid-free apoA-I caused a small but not significant decrease (Fig. 6).

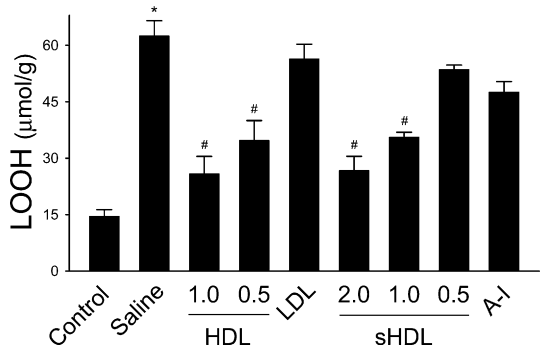

Fig. 6. Effect of HDL on cardiac LOOH production. LOOH content was measured in non-ischemic hearts (Control) and in I/R hearts treated with saline, HDL, LDL (1 mg/ml), sHDL or lipid-free apoA-I (1 mg/ml) during the 10 min immediately before ischemia. Data are mean ± SEM, $n = 3$ for each group. $^{*}P < 0.05$ versus control; $^{\#}P < 0.05$ versus saline.

4. Discussion

The present study demonstrates that both plasma-derived and synthetic HDL attenuate the I/R-induced activation and subsequent release of cardiac MMP-2. The prevention of MMP-2 activation is dose-dependent, occurs at physiological HDL concentrations, and is specific for HDL, as LDL had no effect. The preservation of the cardiac MMP-2 content by HDL parallels the improvement of post-ischemic contractile function and the reduction of cardiac damage, indicating that prevention of MMP-2 activation represents a novel mechanism for HDL-mediated cardioprotection.

MMP-2 is synthesized as a pro-enzyme, which is activated extracellularly by proteolytic cleavage of the inhibitory pro-peptide domain [1]. ROS also directly activate MMP-2 by oxidizing the sulphydril bond between a cysteine residue of the pro-domain and the Zn^{2+} catalytic center, resulting in partial enzyme activation followed by an intramolecular cleavage of the pro-peptide [1]. The I/R-induced ROS formation, which occurs within the first minutes of reperfusion [14], is likely responsible for the rapid MMP-2 activation in I/R hearts [15]. Active MMP-2 then contributes to early contractile dysfunction [5], through the degradation of intracellular proteins, such as TnI [4], before being released from the heart. MMP activation may also contribute to myocardial I/R injury in humans, as demonstrated in patients undergoing coronary bypass grafting with cardio-pulmonary bypass [16].

HDL are complex macromolecules with a number of activities in lipid transport and modulation of endothelial function. In addition, HDL exert a variety of anti-oxidant effects, which may explain the inhibition of I/R-induced MMP-2 activation observed in the present study. HDL were shown to reduce ROS generation in a cell-free system [17], in cultured vascular smooth muscle cells exposed to oxidized LDL [8], and in the carotids of rabbits undergoing periarterial manipulation [18]. In the present study, the treatment with plasma-derived or synthetic HDL significantly reduced the I/R-induced ROS burst, as demonstrated by the decrease of cardiac LOOH content, fully consistent with a previous study in a similar experimental setting [19]. This effect may result either from inhibition of ROS biosynthesis, by, e.g., chelation of transition metals [20], or from enhanced ROS degradation, mediated by intracellular catalase [8], or HDL-carried paraoxonase [21]. Not all these mechanisms are shared by sHDL and lipid-free apoA-I, which certainly lack paraoxonase activity and do not chelate transition metals [20], thus justifying the lower activity vs. plasma-derived HDL in preventing hydroperoxide formation and consequent MMP-2 activation.

The direct cardioprotection by HDL is thus the result of multiple mechanisms, going from prevention of MMP-2 activation, to TNFα inactivation and stimulation of prostaglandin release [6]. All together, these multiple mechanisms add to other HDL functions in explaining the association of a low-HDL phenotype with both short-term and long-term adverse prognosis after an acute coronary event, and provide the rationale for the therapeutic use of HDL-derived products in a variety of cardiovascular diseases.

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